

Persistence of an intact endometrial matrix and vessels structure in women exposed to VA-2914, a selective progesterone receptor modulator

Endometrial vessels in VA-2914 exposed women

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ABSTRACT

Background: VA-2914 is a selective progesterone receptor modulator with potential contraceptive activity which induces amenorrhea while progestins cause endometrial spotting and bleeding. This abnormal bleeding due to progestins is a consequence of focal stromal proteolysis by increase in naked vessel size and density.

Our objective was to quantify the effects of VA-2914 on endometrial vascularisation, fibrillar matrix and VEGF-A expression in endometrial biopsies from 41 women before and after 12 weeks daily treatment with a placebo or 2.5 mg, 5 mg or 10 mg VA-2914.

Methods: Collagen fibrillar network was stained by silver impregnation. Vessel area, density and structure were quantified with a computer-assisted image analysis system after double immunostaining using an anti-von Willebrand Factor (endothelial cells) and an anti-alpha Smooth Muscle Actin (vascular smooth muscle cells) marker antibody. VEGF-A mRNA were quantified by RT-PCR and localized by immunohistochemistry.

Results: The endometrial vessels, collagen network and mRNA levels of VEGF-A were identical during the luteal phase at baseline and in VA-2914 treated women. VEGF-A distribution was unchanged.

Conclusions: VA-2914 doesn't alter the endometrial matrix and cells and does not modify the endometrial vessel morphology as compared to baseline biopsies.

Introduction

Ovarian estrogens induce endometrial proliferation during the proliferative phase of the menstrual cycle. After ovulation, progesterone induces glandular secretion and stromal (pre)-decidualization. In the absence of embryo implantation, the fall of both ovarian steroids in the late secretory phase triggers a cascade of events leading to vascular damage, proteolytic breakdown, shedding of the functional layer and menstrual bleeding. Irregular extracellular matrix breakdown and bleeding between menstruations are common disorders that occur in diverse endometrial pathologies or upon progestin treatment (1).

Progestin administration causes endometrial vessel remodelling characterized by an increased number of dilated and “naked” vessels devoid of pericytes that are associated with endometrial bleeding (2).

Progesterone receptor ligands include not only pure agonists or antagonists (PA) but also compounds with mixed activity. They are all belonging to the class of selective progesterone receptor modulators (PRMs). Many PRMs display direct antiproliferative effects in the endometrium justifying their possible use in the treatment of endometriosis and myoma associated bleeding. They also suppress late follicular development, block the LH surge and retard endometrial maturation, which renders them potential estrogen-free contraceptive drugs (3).

VA-2914 is a steroid derived from 19-norprogesterone with potent *in vitro* and *in vivo* progesterone antagonist activity (4,5). Acute and chronic toxicology studies in animals indicate a satisfactory safety profile, with considerably less antiglucocorticoid activity in cell culture (4) and in the rat (6) than that of mifepristone. It inhibits ovulation in rats in a dose-dependent manner upon single-dose oral administration and exhibits, by a decrease in implantation sites, an antifertility activity during continuous low-dose administration (7). VA-2914 is also effective as postcoital contraception in animal models (6,7) and in women (8).

A series of Phase I clinical studies has been performed to determine the effects of VA-2914 on ovulation and endometrial maturation in women (9). We recently evaluated the effects of VA-2914, administrated continuously for 3 months, on ovulation and endometrial maturation in 46 normal women. Anovulation was observed in 80% of women in the 5 and 10 mg groups. Plasma estradiol levels remained in the physiological follicular phase range throughout treatment. No endometrial hyperplasia was noted. Amenorrhea occurred in 82 and 90% of women in the 5 and 10 mg groups (10).

In this study, we evaluate the effects of low dose VA-2914 on the degradation of the endometrial matrix as well as on endometrial vessel characteristics and on the expression of VEGF-A, an angiogenic cytokine known to play a key role on blood vessel growth and maturation (11).

Materials and Methods

Patients

The study was approved by the Ethics Committees at the University of Liege and Saint-Antoine Hospital of Paris. Written informed consent was obtained from each subject. 46 women, aged 18-35 years, with documented regular ovulatory menstrual cycles (25-32 days), within 20% of ideal body weight, not currently using an intrauterine device, and not having used reproductive hormonal medications or glucocorticoids for at least 12 weeks before the study, were randomly assigned to either a placebo or one of the three treatment groups (2.5 mg, 5 mg, or 10 mg/d VA-2914 - HRA Pharma, Paris, France). The therapy was started on the first or second day of menstrual bleeding following a baseline observational cycle and taken daily for 12 weeks. Women agreed to prevent pregnancy by abstinence or use of barrier contraception, or they were protected by tubal ligation or partner vasectomy. An endometrial biopsy was performed on day 6 to 8 after the LH surge determined by an urinary test during baseline cycle and treatment month 3. Endometrial biopsy was performed on treatment day 77 in the absence of detectable urinary LH surge during treatment month 3.

Hormonal analysis

E2 was measured by a direct RIA using Diasorin reagents (Anthony, France). The cross reactivity of estrone and estriol was only 0.6%. CVs at the level of 50 pmol/l were 3.6 and 10.4 respectively, and at the level of 260 pmol/l, 2.9 and 6.8%, respectively.

Progesterone was measured by RIA using CIS biointernational reagents (Gif sur Yvette, France). The only significant cross reacting steroids were: deoxycorticosterone 6.2%, 20alpha-dihydroprogesterone 2.2% and 6beta-dihydroprogesterone 2.1%. CVs at the level of 10 ng/ml were 3.5 and 4.5, respectively, and at the level of 18.8 ng/ml, 4.4 and 4%, respectively (10).

Endometrial tissues

Human endometrial biopsies were obtained from 41 women who completed the study with a Cornier Pipelle suction curette (C.C.D. International, Paris, France). A fragment was immediately snap frozen and stored in liquid nitrogen until analysis. The other part of the biopsy was fixed in 4% formaldehyde solution in phosphate buffer saline (PBS) and embedded in paraffin. Histological sections were stained with hematoxylin and eosin. Specimens were evaluated in a blinded fashion by two independent pathologists who classified them as proliferative or early (days 17-19), mid (days 20-22) or late (days 24-26) secretory according to classical criteria (12). The immunohistology and

quantitative analysis were performed on entire tissue section (5µm thick) with a mean size of 25 mm² and with a mean number of gland sections of 100.

Von Willebrand Factor/alpha-Smooth Muscle Actin double immunostaining

Serial sections were mounted on silanized slides and used for immunohistochemistry. Endometrial vessels were identified by double immunostaining of endothelial cells and smooth muscle cells. The primary antibodies (Abs) used were a rabbit polyclonal anti-human von Willebrand Factor (vWF, A082, Dako, Denmark) and a mouse monoclonal anti-alpha Smooth Muscle Actin (αSMA, clone 1A4, A2547, Sigma-Aldrich, USA). Slides were washed in Tris-HCl, pH 7.6 between all steps unless otherwise stated. Endogenous peroxidase was blocked with 3% H₂O₂ for 20 min at room temperature (RT). Slides were incubated with normal sheep serum (NSS) (Hormonology laboratory, Marloie, Belgium) for 30 min at RT, directly followed by vWF antibody (1/500 in NSS/Tris 10%) overnight at 4°C. A swine anti-rabbit immunoglobulin conjugated to peroxidase (P0217, Dako, Denmark) was used as a secondary antibody. DAB+ (Liquid DAB+ substrate chromogen system, K3468, Dako, Denmark) was applied for 15 min at RT in the dark as a chromogen and sections were rinsed in H₂O. Slides were incubated with normal goat serum (NGS) (Hormonology laboratory, Marloie, Belgium) before incubation with the second primary antibody, αSMA (1/400 in NGS/Tris 10%) for 90 min at 37°C. A goat anti-mouse Ab conjugated to biotin (E 0433, Dako, Denmark) diluted 1/400 in Tris buffer for 30 min at RT was used as a secondary Ab, followed by an incubation for 30 min at RT with streptavidin-alkaline phosphatase (D 0396, Dako, Denmark) diluted 1/500. Finally, Fast Red chromogen system (K4016, Dako, Denmark) was applied for 10 min at RT in the dark. Sections were rinsed in H₂O and mounted in Aqua Polymount (Polysciences, Inc., Warrington, PA). Negative control was performed by replacing each primary antibody with normal serum.

VEGF-A immunohistochemistry

VEGF-A protein was localized in sections of paraffin-embedded endometrial biopsies. Sections were stained with VEGF-A antibody by the following steps. After dewaxing and rehydrating, tissue sections were subjected to antigen retrieval in pressure cook at 1.4 bar and 126°C for 11 min using citrate buffer and were then allowed to cool for 20 min. Endogenous peroxidase were blocked in 3% H₂O₂ for 20 min at RT and non-specific binding of the primary antibody was blocked with 10% BSA. Incubation with rabbit polyclonal anti-human VEGF-A Ab (A-20, Santa Cruz Biotechnology, CA, USA) diluted 1/50 was conducted 60 min at RT. Sections were washed in PBS (5x 5 min) before incubation with the secondary Ab (goat anti-rabbit ENVISION/HRP, K4003, DAKO, Denmark) for 30 min at RT. Finally, staining was revealed with DAB system and sections were counterstained with haematoxylin.

Location and intensity of immunostaining were measured using a semi quantitative scoring system. This scoring system is a standard method used in previous studies (13-16), and a high correlation has been demonstrated between objectively measured immunoreactivity (image analysis) and semi-quantitative scoring of immunostaining patterns (16). Immunostaining intensity and distribution of epitopes in all tissue sections were assessed on a four-point scale: 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining and expressed as a mean score and range.

VEGF-A mRNA expression

Frozen tissue was pulverized using a Dismembrator (B. Braun Biotech International, GmbH, Melsungen, Germany) and suspended in lysis buffer (5 M guanidine thiocyanate, 25 mM sodium citrate, 17 mM *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol [pH 7]). Total RNA was purified by 'Qiagen' method (Qiagen Benelux). The concentration and purity of RNA was determined by spectrophotometry. RT-PCR amplification was performed using GeneAmp ThermoStable rTth reverse transcriptase RNA PCR kit (Perkin-Elmer, Branchburg, NJ) and the following specific pair of primers: *VEGF-A*: 5'-TCTACCTCCACCATGCCAAGT-3' and 5'-GCTGCGCTGATAGACATCCA-3' (104 base pair product) and *28S*: 5'-GTTACCCCACTAATAGGGAACGTGA-3' and 5'-GATTCTGACTTAGAGGCGTTCAGT-3' (212 base pair product). Reverse transcription was performed from 10ng RNA total at 70°C for 15 minutes, followed by 2 min at 95°C to denature DNA. PCR amplification was run as follows: 15 sec at 94°C, 30 sec at 60°C and 15 sec at 72°C during 31 cycles for *VEGF-A* and 15 sec at 94°C, 20 sec at 68°C, and 10 sec at 72°C during 15 cycles for *28S*, and followed by a final 2 min extension step at 72°C. RT-PCR products were resolved on 10% polyacrylamide gels and analysed using a Fluor-S Multimager (BioRad, Hercules, CA) after staining with Gelstar dye (FMC BioProducts, Rockland, ME). Products were quantified by normalization with respect to *28S* ribosomal RNA. The levels of expression were compared at baseline and in a combined group of women exposed to various doses of VA-2914.

Staining of the argyrophilic fibrillar network

Silver impregnation of histological sections was performed as previously described (17).

Image analysis and measurement

Slides were observed with an Olympus microscope (Omnilabo, Aartselaar, Belgium) and the entire section (Mean area 25 mm²) was analysed at magnification of 400X. Vessels were identified by positive von Willebrand factor staining and the presence of pericytes was documented according to the α SMA staining as previously described in details (2). Briefly, a coating of the endothelial cells with

α SMA positive cells (pericytes) was considered as terminal venules or arterioles, while ‘naked’ vessels were considered to be capillaries. A score (0 or 1) was attributed to blood vessels according to absence or presence of α SMA staining. Vessels were counted in the entire biopsy.

The contour of each vessel type was drawn manually at 400X magnification using Photoshop software by two different colours: blue for α SMA negative vessels (score 0), red for α SMA stained vessels (score 1) and green for tissue boundaries (figure 1A). Then, the colour image was decomposed into its three components: red (R), green (G) and blue (B) (18). Figures 1 B and C show binary images of vessels scored 0 and 1, respectively.

The area of each vessel section and the relative vascular area, defined as the ratio of vessel section surface to total tissue area, were measured in each binary image. Image processing and measurements were performed with the software Aphelion 3.2 from Adsis (France) on a PC.

Statistical analysis

The parameters were expressed in terms of the mean \pm standard error. Analyses for statistical significance were evaluated by Kruskal-Wallis test with Dunn’s correction for multiple comparisons. Then, for comparison between two groups, Mann-Whitney U test and paired Wilcoxon were used. Statistical significance was set at $p < 0.05$.

Results

Patients and endometrial histology

Patient’s characteristics and endometrial histology are described in Table I and have been reported in details elsewhere (10). Briefly, all women in the placebo group and most patients (9/11) exposed to VA-2914 2.5 mg/d experienced normal menstrual cycles, while amenorrhea was observed in majority of women receiving 5 or 10 mg/d (Table I). During the baseline cycle, thirty-two biopsies showed typical features of the mid-secretory phase and one showed a polyp. No tissue could be obtained from 8 women. After 12 weeks of treatment with VA-2914, 17 biopsies were classified as a secretory pattern and 1 as proliferative. No tissue was obtained from 11 patients. Patients with polyps were excluded for further analysis.

The histological features of endometria in women at baseline during the mid secretory phase (figure 2A) and after 12 weeks of continuous treatment with VA-2914 (2.5-10 mg/d) (figures 2B-D) were similar.

Estradiol and progesterone plasma levels during the third treatment month are described in Table II. All women in the placebo treated group exhibited an endometrial secretory pattern with E2 and progesterone plasma levels typical of a mid luteal phase. Among the 6 women exposed to VA-

2914 2.5 mg/d, 3 had progesterone plasma levels above 6 ng/ml indicative of an ovulation and their endometrium also showed the expected secretory pattern. Three other women in that group had progesterone plasma levels below the range of an ovulatory cycle (respectively 0, 0 and 2 ng/ml). In 2 of them an endometrial pattern of a secretory phase was documented. Among the 13 women exposed to VA-2914 5 or 10 mg/d, only 3 had progesterone plasma levels above 6 ng/ml (respectively 24, 16 and 13 ng/ml). In the 10 other women, progesterone plasma levels was below the detection limit. The histological demonstration of a secretory pattern in these women should therefore be ascribed to a VA-2914 injury. In all women, the estradiol plasma levels were in the range of a spontaneous mid follicular phase. Altogether these data suggest that exposure to 5-10 mg/d VA-2914 blocks ovulation but does not induce the histological changes classically elicited by anti-gonadotropic progestins.

Stromal breakdown and fibre lysis

Staining of argyrophilic fibrillar network documented the integrity of the collagen-rich fibrillar network in all biopsies at baseline and after treatment with placebo or all doses of VA-2914 (figures 2E-F). This is in sharp contrast with the stromal breakdown and extensive endometrial matrix proteolysis in women with progestin-only contraceptives (1,19). This indicates that VA-2914 does not elicit the extensive endometrial stromal breakdown observed after progestin exposure.

Microvessel density and area

Altogether more than 12,000 vessel sections were counted and characterized. Capillaries were identified by the isolated presence of vWF positive endothelial cells (figure 2G) while a continuous layer of α SMA positive cells coating the endothelial cells characterized small arterioles and venules (figure 2H).

The relative vascular area (ratio of vessel section surface to total tissue area) was determined in endometrial biopsies of 32 women at baseline during the mid-secretory phase and after treatment with VA2914 (figures 3A-B). At baseline, about 80% of the vascular areas were occupied by vessels coated with α SMA positive cells. After 12 weeks treatment with various doses of VA-2914, this proportion remained unchanged (figure 3B).

The mean vessel section areas were determined by computer assisted method (figure 3C). Vessels coated with smooth muscle cells were larger than “naked” capillaries in all groups. Their respective sizes were identical at baseline and during treatment with VA-2914. No difference in vessels counts, density and size was observed with a Mann-Whitney test. The comparison of values from each individual woman at baseline and after treatment by a paired Wilcoxon test failed also to show a significant difference.

VEGF-A immunolocalization

During the baseline cycle, VEGF-A was strongly localized in the apical part of the cytoplasm of the surface epithelium [score 2.5, (1-3)] and of glandular epithelial cells [score 2.9, (2-3)]. Mild expression was also found in the stroma [score 0.8, (0-1)] and endothelial cells [score 0.8, (0-1)] (Table III, figure 2I). VEGF-A localization remained unchanged in all cellular compartments following 12 weeks of treatment with placebo or VA-2914 (Table III, figure 2J).

VEGF-A expression

The mRNA expression of VEGF-A was analysed by RT-PCR in endometrial tissue from 11 women at baseline and from 8 women treated with various doses of VA-2914. The VEGF-A mRNA levels remained unchanged (data not shown).

Discussion

VA-2914 is a new PRM that is undergoing clinical testing. Endometrial changes associated with a minimum of three months of chronic treatment with PRMs including VA-2914 were recently evaluated by a panel of pathologists. Biopsies exhibited an unusual architecture characterized as glandular dilatation and little evidence of mitosis, consistent with the proposed antiproliferative effect of PRMs (20). The specific aspect observed in endometrial biopsies during PRM treatment are now described as PRMs Associated Endometrial Changes (PAEC) (21). In our study, the endometria were mostly secretory at doses of 2.5 and 5 mg VA-2914 independently of E2 and progesterone levels (Table II). Atrophy has been described at 10 mg dosage (Table I). In this study, respectively 82 and 90% of women became amenorrheic during the 12 weeks daily treatment with 5 or 10 mg (see also (10)). Although a comparison between endometria after treatment with oral VA-2914 and after treatment with an oral progestin such as oral levonorgestrel is an appropriate design for a subsequent clinical study, this phase IIA protocol aimed at evaluating in a limited set of women the impact of VA-2914 upon the endometrial histology, ovulation and endocrine ovarian activity in women with documented spontaneous ovulatory cycle. This first clinical study indicates that a 12 weeks exposure to VA-2914 5 or 10 mg/d causes anovulation, amenorrhea and a persistent estradiol secretion with an endometrial pattern that closely resembles that of a spontaneous ovulatory cycle during the mid luteal phase. This pattern is strikingly distinct from that observed with other PRMs that cause endometrial hyperplastic changes which are worrisome for chronic administration (22).

Argyrophilic staining of the collagen fibrillar network showed no evidence of endometrial matrix degradation during the mid-secretory phase of spontaneous ovulatory cycle (control) or in women exposed to VA-2914. Focal stromal breakdown, i.e. menstrual-like tissue collapse and

fragmentation, and lysis of the collagen-rich argyrophilic fibrillar network are classically observed in endometrial biopsies performed at menstruation or at the start of a bleeding episode in women receiving subcutaneous or intrauterine levonorgestrel as progestin (1). Such focal and stromal breakdown and collagen fiber lysis are evidenced in bleeding endometria and associated with higher activities of collagenase-1 and gelatinases A and B together with lower tissue inhibitor of metalloproteinase-1 than in non-bleeding endometria (1,19). The absence of matrix remodelling and collagen lysis in normal secretory endometria and in women receiving VA-2914 suggests that the endometrial changes elicited by menstruation and/or prolonged progestin exposure are absent in our patients.

We have shown previously that extensive endometrial remodelling was induced by exogenous LNG administration in the form of an intrauterine system (2). Endometrial samples obtained in 8 women exposed for 1 month to LNG-IUS displayed an 11.5-fold increase in small naked vessel number and a 6-fold increase in pericytes coated vessels. In women using long-term LNG-IUS, a 4-fold increase in the number and size of fragile capillaries (2). We hypothesized that such increased number of fragile “naked” capillaries could easily be injured and responsible for bleeding. We therefore compared in this study the impact of VA-2914 or of endogenous progesterone during the mid-secretory phase of spontaneous ovulatory cycle (baseline cycle) on the pattern of endometrial vessels. Endometrial VA-2914 exposed women exhibited identical blood vessel number, area, density and maturation in comparison to the pattern of normal spontaneous secretory endometrium under the influence of endogenous estradiol + progesterone. This pattern of endometrial vessels was observed independently of the endogenous levels of E2 and progesterone (Table II). In the same patients, we documented previously the persistence of physiological estradiol levels (10). The similarity of pattern in the presence of endogenous progesterone or of VA-2914 is somewhat surprising since VA-2914 is not a progestin but a selective modulator of the progesterone receptor. This pattern may contribute to explain the high level of amenorrhea observed in our patients. Additional clinical study comparing the impact of orally given synthetic progestin in the presence or absence of VA-2914 will be necessary to more precisely delimit the impact of this new drug on the endometrial physiology.

The presence of VEGF-A mRNA and protein have been demonstrated in the human endometrium throughout the menstrual cycle (23). Most studies including ours, have shown predominant localization of VEGF-A in the apical part of surface and glandular epithelial cells compared to the stroma (figure 1E and (24)). In this study, VA-2914 did not modify the VEGF-A distribution nor its relative staining intensity. This PRM thus acts in a way distinct from that of mifepristone which increases microvessel density and decreases stromal VEGF in endometria of women exposed for 120 days to mifepristone (13).

Altogether, this preliminary phase II study performed on a limited number (46) of women confirms that the PRM VA-2914 maintains matrix and vessel stability, does not impair the expression of the angiogenic cytokine, VEGF-A, and allows the maintenance of a stable vascular network that

closely resembles that observed during the secretory phase of spontaneous ovulatory cycle despite anovulation and amenorrhea. This drug could, in this way, be associated with an improved clinical tolerance profile since it is devoid of the endometrial matrix and vascular remodelling associated with menstruation or with the bleeding conveyed by withdrawal of progesterone.

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Figure legends

Figure 1: Image analysis and vessel quantification. (A) The contour of each type of vessel was drawn manually in the original image by the following different colors: blue for vessels scored 0 and red for vessels scored 1. Green delineates the area of tissue in which measurements are performed. (B) Binary images of score 0 vessels and (C) binary image of score 1 vessels contained in the original image.

Figure 2: (A-D) Representative fields of hematoxylin-eosin-stained histological sections of secretory endometrium during baseline cycle (A) and after 12 weeks with 2.5 mg/d (B), 5 mg/d (C) and 10 mg/d VA-2914. Original magnification 200X. (E-F) Stromal collagen-rich argyrophilic fibrillar network using silver impregnation. Endometrium after 12 weeks treatment with placebo (E) and 10 mg/d VA-2914 (F). Original magnification, 200X. (G-H) Immunolocalization of alpha-Smooth Muscle Actin (α SMA), von Willebrand Factor (vWF) and VEGF in endometrium. In the double immunostaining, α SMA positive cells (pericytes) were labelled in red and vWF positive cells (endothelial cells) were stained in brown. α SMA negative vessels (G), vessels with continuous layer of α SMA positive perivascular cells (H). Original magnification 400X. (I-J) Immunolocalization of VEGF-A in endometrial glands (Gl.), stroma (Str.), surface epithelium (Surf.) and vascular endothelium (VE) during the baseline cycle (I) and after treatment with 10 mg/d VA-2914 (J). The VEGF-A distribution was identical in endometrium of women treated with 2.5 or 5 mg/d VA-2914 (data not shown). Original magnification, 200X.

Figure 3: Quantification of vessel types in endometrium at baseline or after 12 weeks treatment with VA-2914. (A) The relative vascular area represents the percentage of total tissue area occupied by vessels. It is calculated as the ratio between the vessel section surfaces

x 100 to total tissue area. (B) The relative proportion of vessel types refers to the percentage of naked and pericytes coated vessels. (C) The mean vessel section area (μm^2) represents the mean absolute area occupied by both types of vessels. These parameters were determined by computer assisted method for 'naked' capillaries (vWF+ only) and smooth muscle cells coated vessels (vWF+ and $\alpha\text{SMA}+$) in endometrium collected during baseline cycle (n=32) and after 12 weeks treatment with VA-2914 at doses of 2.5 mg (n=6), 5mg (n=10) and 10 mg (n=3) ($p>0.05$). The vessel parameters of endometrium from women exposed to placebo (n=5) were identical to those of the baseline group (n=32) (data not shown).

Table I: Patient's characteristics and endometrial histology at baseline cycle and during the third treatment cycle. The patient number "n" refers to the number of women in each treatment group.

	<i>Baseline cycle</i>	<i>Placebo</i>	<i>VA-2914 2.5 mg/d</i>	<i>VA-2914 5 mg/d</i>	<i>VA-2914 10 mg/d</i>
PATIENTS, <i>n</i>	41	9	11	12	9
Age, <i>Mean ± SD</i>	26.0 ± 5.2	26.1 ± 3.9	23.8 ± 5.4	27.6 ± 5.2	26.4 ± 6.0
Amenorrhea, <i>n</i>	0	0	2	9	9
HISTOLOGY, <i>n</i>					
Proliferative	0	2	1	0	0
Secretory	32	5	5	10	2
Polyp	1	0	0	1	1
Atrophic	0	2	0	0	1
Absence of tissue	8	0	5	1	5

Table II: Estradiol and progesterone plasma levels during the third treatment month with placebo or 2.5 mg/d, 5 mg/d and 10 mg/d VA-2914.

<i>Treatment groups</i>	<i>Patients</i>	<i>Endometrial characteristics</i>	<i>E2 (pg/ml)</i>	<i>Progesterone (ng/ml)</i>
Placebo	1	late secretory (day 24)	80	20
	2	early secretory (day 18)	180	18
	3	mid secretory (day 20)	70	14
	4	mid secretory (day 20)	60	21
	5	early secretory (day 18)	120	10
2.5 mg/d VA-2914	1	mid secretory (day 20)	160	18
	2	early secretory (day 18)	100	9
	3	proliferative	200	0
	4	early secretory (day 18)	120	10
	5	mid secretory (day 20)	40	0
	6	mid secretory (day 20)	100	2
5 mg/d VA-2914	1	early secretory (day 18)	280	24
	2	mid secretory (day 20)	150	1
	3	mid secretory (day 20)	90	0
	4	mid secretory (day 20)	30	0
	5	late secretory (day 25)	50	0
	6	late secretory (day 25)	40	0
	7	late secretory (day 28)	30	16
	8	late secretory (day 23)	110	0
	9	late secretory (day 25)	260	0
	10	mid secretory (day 20)	40	0
10 mg/d VA-2914	1	mid secretory (day 20)	200	0
	2	mid secretory (day 20)	160	13
	3	mid secretory (day 20)	40	0

Table III: Semiquantitative evaluation of VEGF-A immunolocalisation in endometrium during baseline and treatment cycles. Mean score (median) (p>0.05).

(0: absence of staining, 1: staining of <25% of the surface epithelium, or glands, or stroma, or endothelium, 2: staining between 25% and 50% of the specific structures and 3: strong >50% of the structures studied.)

	<i>Baseline cycle (n=32)</i>	<i>3rd treatment cycle</i>			
		<i>Placebo (n=5)</i>	<i>VA-2914 2.5 mg/d (n=6)</i>	<i>VA-2914 5 mg/d (n=10)</i>	<i>VA-2914 10 mg/d (n=3)</i>
Surface epithelium	2.5 (3)	1.8 (2)	3 (3)	2.8 (3)	3 (3)
Glands	2.9 (3)	2 (2)	3 (3)	2.8 (3)	3 (3)
Stroma	0.8 (1)	0.5 (0.5)	0.7 (1)	0.7 (1)	1 (1)
Endothelium	0.8 (1)	0.6 (1)	0.7 (1)	0.5 (0.5)	1 (1)



Figure 1

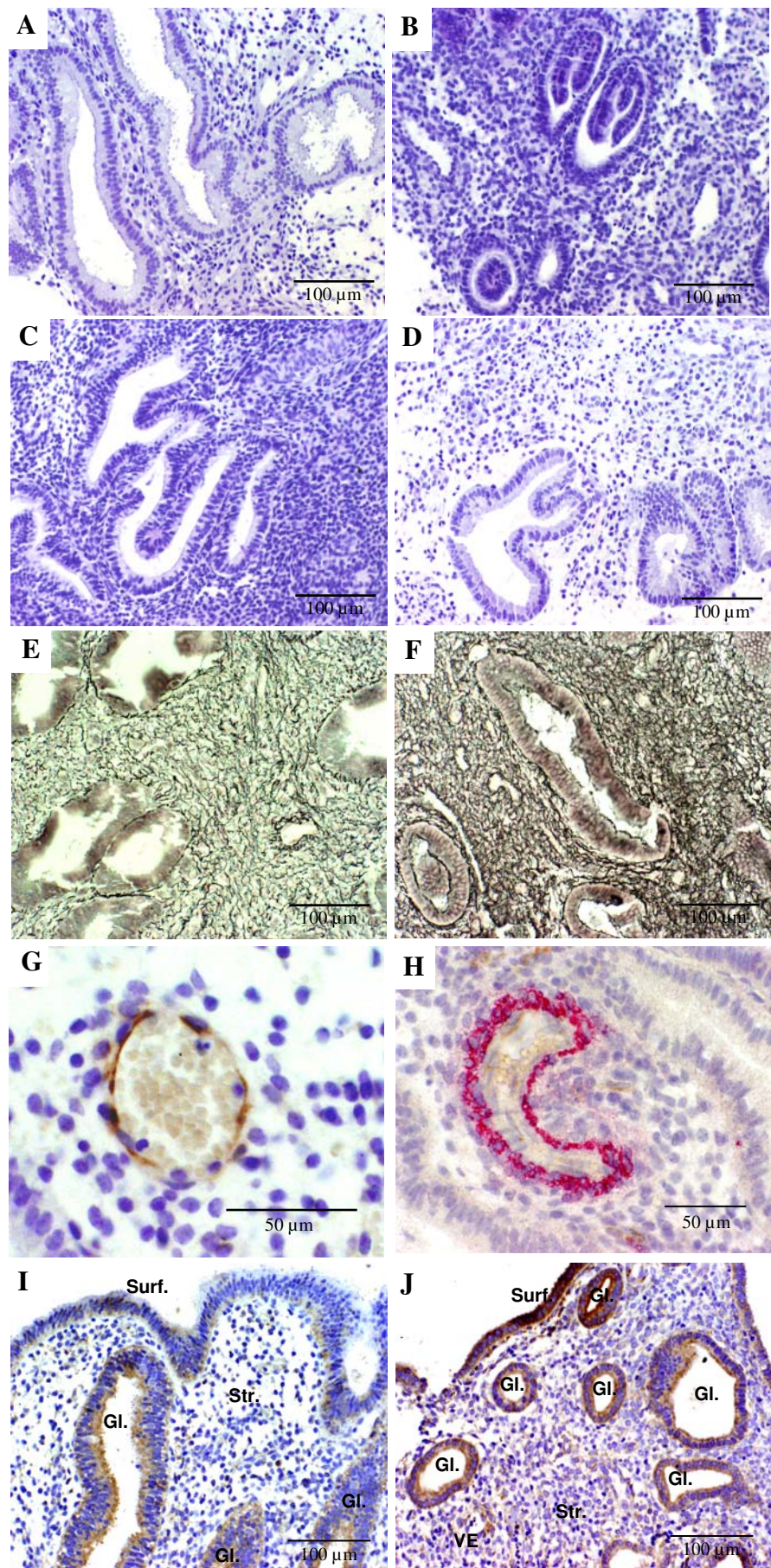


Figure 2

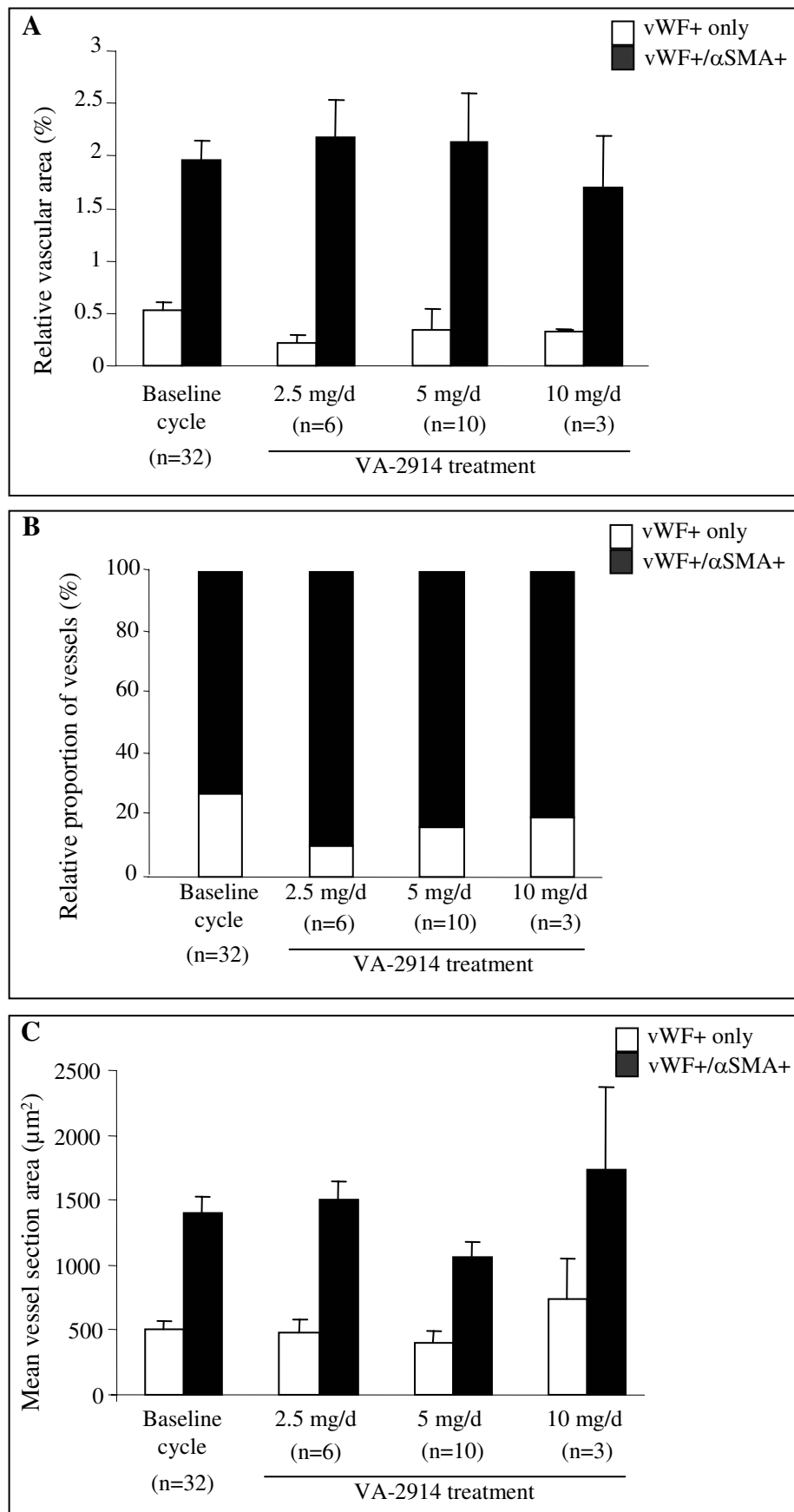


Figure 3